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Detection of *Renibacterium salmoninarum*, the Causative Agent of Bacterial Kidney Disease in Salmonid Fish, from Pen-Cultured Coho Salmon

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The detection of *Renibacterium salmoninarum* antigen from pen-cultured coho salmon was attempted. Flounder (*Limanda herzensteini*) (n = 24), greenling (*Hexagrammos otakii*) (n = 5), Japanese sculpin (*Cottus japonicus*) (n = 1), and flathead (*Platycephalus indicus*) (n = 22) captured by fishing around coho salmon net pens were examined for the presence of *R. salmoninarum* antigen by an indirect dot blot assay and by an indirect fluorescent-antibody technique using polyclonal and monoclonal antibodies. *R. salmoninarum* antigen was detected from kidney samples of one greenling and six flathead. Moreover, 86 scallops (*Patinopecten yessoensis*) were hung from the edge of the net pen for 50 days, and *R. salmoninarum* antigen was demonstrated in 31 samples by the indirect dot blot assay and the indirect fluorescent-antibody technique.

Bacterial kidney disease (BKD) has been reported as the cause of serious mortality among salmonid fish cultured in fresh water (7). The causative agent of BKD is a gram-positive, nonmotile, non-spore-forming diplobacillus and belonged to the genus *Corynebacterium* (9). Research by Sanders and Fryer (13) on the guanosine-cytosine (GC) content, peptidoglycan, and cell wall composition of the bacterium prompted them to place it in a new genus, *Renibacterium*, and give it the species name *salmoninarum*. The first appearance of BKD in Japan was reported from Hokkaido in 1973 (8), and it spread rapidly among Japanese salmonid fish farms. Clinical signs of this disease are gray-white necrotic abscesses in the kidney, exophthalmia, and abdominal distension. The infection progresses slowly, and a long period is required between initial infection of fish and the appearance of clinical signs (7). It is generally accepted that horizontal transmission is an important means of spreading BKD, although vertical transmission also occurs. Austin and Rayment (2) reported that this bacterium can be isolated from feces and sediment in freshwater tanks in which BKD-infected rainbow trout, *Oncorhynchus mykiss*, were cultured. BKD also occurs in salmon after they are transferred from fresh water to seawater. The possibility of horizontal transmission in seawater was suspected (6); however, it has not been demonstrated.

In Japan, the production of cultured coho salmon, *Oncorhynchus kisutch*, reached 14,000 metric tons in 1989. Coho salmon culture begins with eggs imported from the United States, and after hatching, the fry are reared in fresh water for 10 months and then transferred to net cages in seawater. BKD is the most prevalent bacterial disease in these cultured coho salmon, and the loss attributable to BKD in seawater is larger than that in fresh water (10). In spite of the loss of coho salmon in seawater, no epizootiology of *R. salmoninarum* released from the dead fish into the net cage has been investigated. In this study, we attempted to detect *R. salmoninarum* antigen in the aquatic environment of cultured coho salmon.

The study was conducted in the Okkirai Bay, Iwate Prefecture, Japan. In the bay, 557 tons of coho salmon were being cultured during the experiments, which were performed from April to July 1990. One net pen containing about 3,000 coho salmon was used in this study. We could not investigate the prevalence of BKD in the population, but several fish with symptoms of BKD, including gray-white necrotic abscesses in the kidney or exophthalmia, were observed during this period, and *R. salmoninarum* antigen was detected in 36.7% of dead fish.

We captured fish near the coho salmon net pen by angling. Flounder (*Limanda herzensteini*) (n = 24), greenling (*Hexagrammos otakii*) (n = 5), Japanese sculpin (*Cottus japonicus*) (n = 1), and flathead (*Platycephalus indicus*) (n = 22) were collected. These fish were necropsied, and kidney samples were inoculated onto selective medium (SKDM) (L-cysteine hydrochloride, 1 g; fetal calf serum, 100 ml; tryptone, 10 g; yeast extract, 0.5 g; agar, 10 g; cycloheximide, 50 mg; D-cycloserine, 12.5 mg; oxolinic acid, 2.5 mg; polymyxin B sulfate, 25 mg; pH 6.8; each per liter) (1). The kidney smears from the collected fish were prepared on nonfluorescent slides and examined by Gram staining and an indirect fluorescent-antibody test (IFAT) (5). The sampled kidney was also treated by heating, and an indirect dot blot assay (IDBA) was performed (10). Anti-*R. salmoninarum* rabbit polyclonal antibody and monoclonal antibody were used for the IFAT and the IDBA (11). The monoclonal antibody can recognize the 57-kDa protein which is present on all *R. salmoninarum* strains.

We also tried to isolate *R. salmoninarum* from seawater in which coho salmon were cultured. Twenty milliliters of seawater was collected from the net pen and was passed through a 0.45-μm-pore-size Millipore filter (HA). The filter was inoculated onto SKDM (1) and incubated for 30 days at 15°C. Five lots of water samples were examined.

In a separate experiment, 86 scallops (*Patinopecten yessoensis*) which had been cultured at another site were hung for 30 days at the edge of the net pen of the cultured coho salmon. Isolation of *R. salmoninarum* and the detection of its antigen from the midgut gland of these scallops were performed by the methods described above. Before the beginning of this experiment, no *R. salmoninarum* cells were detected in the midgut gland of five scallops in the same

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TABLE 1. Detection of *R. salmoninarum* antigen from fish captured around the coho salmon net pen

Fish species	No. of fish[a]	No. of samples with antigen with:			
		Polyclonal antibody		Monoclonal antibody	
		IFAT	IDBA	IFAT	IDBA
Greenling	5	0	0	0	0
Flathead	22	6	6	6	6
Flounder	24	0	0	0	0
Japanese sculpin	1	1	1	1	1

[a] None of the fish showed clinical signs of BKD.

batch. As a control, eight scallops were immersed into a seawater suspension of 10^5 *R. salmoninarum* cells per ml for 4 h and then were cultured for 30 days in an aquarium at Kitasato University. Two scallops were examined after 2 days and the other scallops were examined 30 days later for the presence of *R. salmoninarum*.

R. salmoninarum antigen could be detected by IFAT and IDBA using polyclonal antibody from six samples of flathead and one of Japanese sculpin (Table 1). In the case of monoclonal antibody, the same samples were also positive for *R. salmoninarum*. However, these antigen-positive fish did not show any signs of BKD. *R. salmoninarum* antigen was not detected in the kidneys of flounder or greenling. No bacteria were isolated on SKDM, nor were *R. salmoninarum* cells observed by Gram staining.

Isolation of *R. salmoninarum* from the seawater was attempted, and several colonies grew on SKDM from each batch. However, by the Gram stain and serum agglutinating test, no colonies were identified as *R. salmoninarum*.

The *R. salmoninarum* antigen was detected, by IFAT and IDBA using the polyclonal antibody, in the midgut gland of 31 samples of scallops (Table 2). With the monoclonal antibody, 25 samples of 31 *R. salmoninarum* polyclonal antibody-positive samples showed a positive reaction by the IFAT and the IDBA. Thus, *R. salmoninarum* antigen was detected in 25 of 86 scallops examined. *R. salmoninarum* isolation from these scallops was also attempted; however, no bacteria identified as *R. salmoninarum* grew.

BKD infection is limited to salmonids, although lamprey (*Lampetra tridentata*) (4), carp (*Cyprinus carpio*) (12), and sablefish (*Anoplopoma fimbria*) (3) have been experimentally infected with *R. salmoninarum*. Sablefish became moribund or died of apparently incomplete infections of *R. salmoninarum* within 50 to 71 days. We detected *R. salmoninarum* antigen from six samples of flathead and one sample of Japanese sculpin; however, bacterial isolation was not accomplished, and these fishes did not show any signs of BKD. The pathogenicity of *R. salmoninarum* to flathead or Japanese sculpin is still unknown. Although flathead and Japanese sculpin are not important in the Japanese fishing industry, they may be carriers of BKD. Thus, these is a need to examine the pathogenicity of *R. salmoninarum* to these fishes.

TABLE 2. Detection of *R. salmoninarum* antigen from the digestive gland of scallops

Scallop group[a]	No. of scallops	No. of samples[b] with antigen with:			
		Polyclonal antibody		Monoclonal antibody	
		IFAT	IDBA	IFAT	IDBA
Expt	86	31	31	25	25
Negative control	10	0	0	0	0
Positive control	8	8	8	8	8

[a] For the negative control group, scallops were examined before the experiment began. For the positive control group, scallops were immersed in 10^5 cells of *R. salmoninarum* per ml.

[b] No samples yielded bacterial isolates on SKDM.

The isolation of *R. salmoninarum* from 20 ml of seawater in which coho salmon were cultured failed because of the inability of SKDM to support growth. Austin and Rayment (2) attempted the isolation of *R. salmoninarum* from fresh water of an aquarium in which rainbow trout were artificially infected with BKD. Because *R. salmoninarum* was not detected in any water samples, they concluded that the bacterium may have had an affinity for organic, particulate matter. In this experiment, organic particulates contained in 20 ml of seawater were collected with filters and the culture of *R. salmoninarum* was attempted. However, the bacterium identified as *R. salmoninarum* was not isolated. To support the suggestion of Austin and Rayment (2), the detection of *R. salmoninarum* from a lot of organic particulates, at least with more than 20 ml of seawater, might be necessary.

It is well known that shellfish take up nutrients by filtration of bacteria, organic matter, or particles in the environment. We speculated that some *R. salmoninarum* cells might be taken up by shellfish if the release of bacteria occurred in the coho salmon net pen culture. *R. salmoninarum* antigen was detected in 31 of 86 scallops hung from the net pen of cultured coho salmon, and no *R. salmoninarum* antigen was detected in the same lot of scallop before hanging. This fact indicates that *R. salmoninarum* cells were released from coho salmon infected by BKD to the seawater environment and were taken up by these scallops.

This study indicates that *R. salmoninarum* is released from BKD-infected coho salmon cultured in net pens and that other animals, such as other fish species or shellfish, inhabiting the environment take in this bacterium by filtration or eating. This suggests that horizontal transmission of BKD occurs in seawater. Evelyn (6) reported that *R. salmoninarum*-free sockeye salmon (*Oncorhynchus nerka*) became infected while they were held for several months in seawater cages adjacent to cages containing BKD-infected salmon. These animals did not show any signs of BKD; however, these findings suggest that *R. salmoninarum* antigen released from coho salmon infected by BKD was taken up by fish and shellfish inhabiting seawater environments and that these animals play an important role in transmission of BKD. In the present investigation, chum salmon fingerlings were released in close proximity to the coho salmon net pen culture in Okkirai Bay, and no loss of chum salmon caused by BKD has been observed. However, it appears possible for *R. salmoninarum* to infect chum salmon populations. Therefore, there is a need to eliminate BKD-infected or carrier coho salmon from the net pen populations.

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